

The activity of compounds that are functional equivalents to a gene product expressed in extra-embryonic tissue such as recombinant hedgehog protein, analogs, derivatives and disassociation products of hedgehog proteins, and agonists of hedgehog protein receptors such as PTC according to the invention, may stimulate hemotopoiesis and vascular growth by acting on cells or tissues from embryos of different ages including fetal cells, fetal peripheral blood and cord blood, as well as on adult hematopoietic stem cells and adult progenitor cells. The invention includes the use of functional peptides of hedgehog protein. The term "functional peptide" as a subclass of a hedgehog compound defined above, is meant to include peptide fragments of the hedgehog protein that are capable of inducing a biological activity that is the same or equivalent to the entire protein (WO 96/16668, incorporated here by reference).

E² "Homology" refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of homology between sequences is a function of the number of matching or homologous positions shared by the sequences. An "unrelated" or "non-homologous" sequence shares less than 40 percent identity, though preferably less than 25 percent with one of vertebrate hh sequences of the present invention.

Homologs of one of the subject *hedgehog* proteins can be generated by mutagenesis such as by discrete point mutation(s) or by truncation. For instance, mutation can give rise to homologs which retain substantially the same, or merely a subset, of the biological activity of the *hh* polypeptide from which it derived. Alternatively, antagonistic forms of the protein can be generated which are able to inhibit the function of the naturally occurring form of the protein, such as by competitively binding to the *hh* receptor.

Peptides referred to herein as having an activity of a vertebrate hh protein are defined as peptides that have an amino acid sequence corresponding to all or a portion of the amino acid sequences of a vertebrate hh protein which have at least one biological activity of a vertebrate hh protein.

The invention further includes hedgehog compounds described in WO 95/18856 and here incorporated by reference, including homologs of hedgehog proteins, recombinant hedgehog proteins, hedgehog encoding nucleic acids, antisense molecules, gene constructs for use in gene therapy including viral vectors known in the art, combinatorial mutants of hedgehog proteins as agonists or antagonists, and antibodies specific for hedgehog protein epitope. These and other compounds may be selected for modulating hematopoiesis and vascular growth according to the assays of the invention.

The *Hedgehog* family of vertebrate inter-cellular signaling molecules described in WO 95/18856 consists of at least four members. Three of these members, herein referred to as Desert *hedgehog* (*Dhh*), Sonic *hedgehog* (*Shh*) and Indian *hedgehog* (*Ihh*), exist in all vertebrates, including fish, birds, and mammals. A fourth member, herein referred to as Moonrat *hedgehog* (*Mhh*), appears specific to fish. According to the appended sequence listing, (see also Table 1) a chicken *Shh* polypeptide is encoded by SEQ ID No: 1; a mouse *Dhh* polypeptide is encoded by SEQ ID No: 2; a mouse *Ihh* polypeptide is encoded by SEQ ID No: 3; a mouse *Shh* polypeptide is encoded by SEQ ID No: 4 a zebrafish *Shh* polypeptide is encoded by SEQ ID No: 5; a human *Shh* polypeptide is encoded by SEQ ID No: 6; and a human *Ihh* polypeptide is encoded by SEQ ID No: 7.

Table 1
Guide to vertebrate *hedgehog* sequences

	Nucleotide	Amino Acid
Chicken <i>Shh</i>	SEQ ID No. 27	SEQ ID No. 34
Mouse <i>Dhh</i>	SEQ ID No. 28	SEQ ID No. 35
Mouse <i>Ihh</i>	SEQ ID No. 29	SEQ ID No. 36
Mouse <i>Shh</i>	SEQ ID No. 30	SEQ ID No. 37
Zebrafish <i>Shh</i>	SEQ ID No. 31	SEQ ID No. 38
Human <i>Shh</i>	SEQ ID No. 32	SEQ ID No. 39
Human <i>Ihh</i>	SEQ ID No. 33	SEQ ID No. 40

SEQ ID No. 41 is the predicted degenerate sequence of the N-terminal portion of vertebrate *hedgehog* proteins.

In certain embodiments, the polypeptide is identical with or homologous to a *Sonic hedgehog* (*Shh*) polypeptide, such as a mouse or human *Shh* represented by SEQ ID Nos: 39 or 37, an avian *Shh* represented by SEQ ID No: 34, or a fish *Shh* represented by SEQ ID No: 38. For instance, the *Shh* polypeptide preferably has an amino acid sequence at least 70% homologous to a polypeptide represented by any of SEQ ID Nos: 34, 37, 38 or 39, though polypeptides with higher sequence homologies of, for example, 80%, 90% or 95% are also contemplated. Exemplary *Shh* proteins are represented by SEQ ID No. 42. The *Shh* polypeptide can comprise a full length protein, such as represented in the sequence listings, or it can comprise a fragment of, for instance, at least 5, 10, 20, 50, 100 or 150 amino acids in length. Preferred *hedgehog* polypeptides include *Shh* sequences corresponding approximately to the natural proteolytic fragments of the *hedgehog* proteins, such as from about Cys-24 through Glu-188, or from about Asn-189 through Ala-475 of the human *Shh* protein, or analogous fragments thereto.

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cont

In other embodiments, the polypeptide is identical with or homologous to an *Indian hedgehog* (*Ihh*) polypeptide, such as a human *Ihh* represented by SEQ ID No: 40, or a mouse *Ihh* represented by SEQ ID No: 36. For instance, the *Ihh* polypeptide preferably has an amino acid sequence at least 70% homologous to a polypeptide represented by either of SEQ ID Nos: 36 or 40, though *Ihh* polypeptides with higher sequence homologies of, for example, 80%, 90% or 95% are also contemplated. The polypeptide can comprise the full length protein represented by in part by these sequences, or it can comprise a fragment of, for instance, at least 5, 10, 20, 50, 100 or 150 amino acids in length. Preferred *Ihh* polypeptides comprise an N-terminal fragment including Arg-1 through Glu-94, or a C-terminal fragment including His-95 through Ser-3312 of the human *Ihh* represented by SEQ ID No: 40, or analogous fragments thereto.

In still further embodiments, the polypeptide is identical with or homologous to a *Desert hedgehog* (*Dhh*) polypeptide, such as a mouse *Dhh* represented by SEQ ID No: 35. For instance, the *Dhh* polypeptide preferably has an amino acid sequence at least 70% homologous to a polypeptide represented by SEQ ID No: 35, though *Dhh* polypeptides with higher sequence homologies of, for example, 80%, 90% or 95% are also contemplated. The polypeptide can comprise the full length protein represented by this sequence, or it can comprise a fragment of, for instance, at least 5, 10, 20, 50, 100 or 150 amino acids in length. Preferred *Dhh* polypeptides comprise *Dhh* sequences corresponding to the N-terminal portion of the protein, e.g. Cys-23

through Asp-189 or Asn-190 through Gly-396 of SEQ ID No: 35, or analogous fragments thereto.

Hedgehog compounds described in WO 95/18856 include polypeptides encoded by a nucleic acid which hybridizes under high or low stringency conditions to a nucleic acid represented by one of SEQ ID Nos: 27-33. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45 °C, followed by a wash of 2.0 x SSC at 50 °C, are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50 °C to a high stringency of about 0.2 x SSC at 50 °C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22 °C, to high stringency conditions at about 65 °C.

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cont

Polypeptides encoded by nucleic acids having a sequence that differs from the nucleotide sequences shown in one of SEQ ID Nos: 27-33 due to degeneracy in the genetic code are also within the scope of the invention. Such nucleic acids encode functionally equivalent peptides (i.e., a peptide having a biological activity of a vertebrate *hh* polypeptide) but differ in sequence from the sequence shown in the sequence listing due to degeneracy in the genetic code. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC each encode histidine) may result in "silent" mutations which do not affect the amino acid sequence of a vertebrate *hh* polypeptide. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject *hh* polypeptides will exist among vertebrates. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding polypeptides having an activity of a vertebrate *hh* polypeptide may exist among individuals of a given species due to natural allelic variation.

Active portions of the vertebrate *hedgehog* proteins encoded by fragments of the nucleic acids are also suitable for use in the present invention. As used herein, a *hedgehog* gene fragment refers to a nucleic acid having fewer nucleotides than the nucleotide sequence

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Cont

encoding the entire amino acid sequence of a vertebrate *hh* protein represented in SEQ ID Nos: 34-40, yet which (preferably) encodes a peptide which retains some biological activity of the full length protein, e.g., the fragment retains the ability to induce formation and differentiation of the head, limbs, lungs, central nervous system (CNS), or mesodermal patterning of developing vertebrate embryo. Nucleic acid fragments within the scope of the present invention include those capable of hybridizing under high or low stringency conditions with nucleic acids from other species for use in screening protocols to detect other *hedgehog* homologs, as well as those capable of hybridizing with nucleic acids from human specimens for use in detecting the presence of a nucleic acid encoding a *hedgehog* protein, including alternate isoforms, e.g., mRNA splicing variants. Nucleic acids within the scope of the invention may also contain linker sequences, modified restriction endonuclease sites and other sequences useful for molecular cloning, expression or purification of recombinant forms of the subject *hh* polypeptides.

Please amend the specification to include the sequence listing filed herewith.

After the claims, please add a separate page including the following abstract:

The replacement paragraphs presented above incorporate changes as indicated by the marked-up versions below.

The activity of compounds that are functional equivalents to a gene product expressed in extra-embryonic tissue such as recombinant hedgehog protein, analogs, derivatives and disassociation products of hedgehog proteins, and agonists of hedgehog protein receptors such as PTC according to the invention, may stimulate hemotopoiesis and vascular growth by acting on cells or tissues from embryos of different ages including fetal cells, fetal peripheral blood and cord blood, as well as on adult hematopoietic stem cells and adult progenitor cells. The invention includes the use of functional peptides of hedgehog protein. The term "functional peptide" as a subclass of a hedgehog compound defined above, is meant to include peptide fragments of the hedgehog protein that are capable of inducing a biological activity that is the same or equivalent to the entire protein (WO 96/16668, incorporated here by reference).

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<u>Chicken Shh</u>	<u>SEQ ID No. 27</u>	<u>SEQ ID No. 34</u>
<u>Mouse Dhh</u>	<u>SEQ ID No. 28</u>	<u>SEQ ID No. 35</u>
<u>Mouse Ihh</u>	<u>SEQ ID No. 29</u>	<u>SEQ ID No. 36</u>
<u>Mouse Shh</u>	<u>SEQ ID No. 30</u>	<u>SEQ ID No. 37</u>
<u>Zebrafish Shh</u>	<u>SEQ ID No. 31</u>	<u>SEQ ID No. 38</u>
<u>Human Shh</u>	<u>SEQ ID No. 32</u>	<u>SEQ ID No. 39</u>
<u>Human Ihh</u>	<u>SEQ ID No. 33</u>	<u>SEQ ID No. 40</u>

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SEQ ID No. 41 is the predicted degenerate sequence of the N-terminal portion of vertebrate *hedgehog* proteins.

In certain embodiments, the polypeptide is identical with or homologous to a *Sonic hedgehog (Shh)* polypeptide, such as a mouse or human *Shh* represented by SEQ ID Nos: 39 or 37, an avian *Shh* represented by SEQ ID No: 34, or a fish *Shh* represented by SEQ ID No: 38. For instance, the *Shh* polypeptide preferably has an amino acid sequence at least 70% homologous to a polypeptide represented by any of SEQ ID Nos: 34, 37, 38 or 39, though polypeptides with higher sequence homologies of, for example, 80%, 90% or 95% are also contemplated. Exemplary *Shh* proteins are represented by SEQ ID No. 42. The *Shh* polypeptide can comprise a full length protein, such as represented in the sequence listings, or it can comprise a fragment of, for instance, at least 5, 10, 20, 50, 100 or 150 amino acids in length. Preferred *hedgehog* polypeptides include *Shh* sequences corresponding approximately to the natural proteolytic fragments of the *hedgehog* proteins, such as from about Cys-24 through Glu-188, or from about Asn-189 through Ala-475 of the human *Shh* protein, or analogous fragments thereto.

In other embodiments, the polypeptide is identical with or homologous to an *Indian hedgehog (Ihh)* polypeptide, such as a human *Ihh* represented by SEQ ID No: 40, or a mouse *Ihh* represented by SEQ ID No: 36. For instance, the *Ihh* polypeptide preferably has an amino acid sequence at least 70% homologous to a polypeptide represented by either of SEQ ID Nos: 36 or 40, though *Ihh* polypeptides with higher sequence homologies of, for example, 80%, 90% or 95% are also contemplated. The polypeptide can comprise the full length protein represented by in part by these sequences, or it can comprise a fragment of, for instance, at least 5, 10, 20, 50, 100 or 150 amino acids in length. Preferred *Ihh* polypeptides comprise an N-terminal fragment including Arg-1 through Glu-94, or a C-terminal fragment including His-95 through Ser-3312 of the human *Ihh* represented by SEQ ID No: 40, or analogous fragments thereto.

In still further embodiments, the polypeptide is identical with or homologous to a *Desert hedgehog (Dhh)* polypeptide, such as a mouse *Dhh* represented by SEQ ID No: 35. For instance, the *Dhh* polypeptide preferably has an amino acid sequence at least 70% homologous to a polypeptide represented by SEQ ID No: 35, though *Dhh* polypeptides with higher sequence homologies of, for example, 80%, 90% or 95% are also contemplated. The polypeptide can

comprise the full length protein represented by this sequence, or it can comprise a fragment of, for instance, at least 5, 10, 20, 50, 100 or 150 amino acids in length. Preferred *Dhh* polypeptides comprise *Dhh* sequences corresponding to the N-terminal portion of the protein, e.g. Cys-23 through Asp-189 or Asn-190 through Gly-396 of SEQ ID No: 35, or analogous fragments thereto.

Hedgehog compounds described in WO 95/18856 include polypeptides encoded by a nucleic acid which hybridizes under high or low stringency conditions to a nucleic acid represented by one of SEQ ID Nos: 27-33. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45 °C, followed by a wash of 2.0 x SSC at 50 °C, are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50 °C to a high stringency of about 0.2 x SSC at 50 °C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22 °C, to high stringency conditions at about 65 °C.

Polypeptides encoded by nucleic acids having a sequence that differs from the nucleotide sequences shown in one of SEQ ID Nos: 27-33 due to degeneracy in the genetic code are also within the scope of the invention. Such nucleic acids encode functionally equivalent peptides (i.e., a peptide having a biological activity of a vertebrate *hh* polypeptide) but differ in sequence from the sequence shown in the sequence listing due to degeneracy in the genetic code. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC each encode histidine) may result in "silent" mutations which do not affect the amino acid sequence of a vertebrate *hh* polypeptide. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject *hh* polypeptides will exist among vertebrates. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding polypeptides having an activity of a vertebrate *hh* polypeptide may exist among individuals of a given species due to natural allelic variation.

Active portions of the vertebrate *hedgehog* proteins encoded by fragments of the nucleic acids are also suitable for use in the present invention the invention. As used herein, a *hedgehog* gene fragment refers to a nucleic acid having fewer nucleotides than the nucleotide sequence encoding the entire amino acid sequence of a vertebrate *hh* protein represented in SEQ ID Nos: 34-40, yet which (preferably) encodes a peptide which retains some biological activity of the full length protein, e.g., the fragment retains the ability to induce formation and differentiation of the head, limbs, lungs, central nervous system (CNS), or mesodermal patterning of developing vertebrate embryo. Nucleic acid fragments within the scope of the present invention include those capable of hybridizing under high or low stringency conditions with nucleic acids from other species for use in screening protocols to detect other *hedgehog* homologs, as well as those capable of hybridizing with nucleic acids from human specimens for use in detecting the presence of a nucleic acid encoding a *hedgehog* protein, including alternate isoforms, e.g., mRNA splicing variants. Nucleic acids within the scope of the invention may also contain linker sequences, modified restriction endonuclease sites and other sequences useful for molecular cloning, expression or purification of recombinant forms of the subject *hh* polypeptides.

In the claims:

For the convenience of the Examiner, all claims being examined, whether or not amended, are presented below.

Please cancel claims 76-81 (as originally numbered) without prejudice.

57. (Amended) A method of stimulating a population of undifferentiated mammalian mesodermally derived cells to undergo hematopoiesis, comprising contacting the cells with a hedgehog compound so as to stimulate the cells to undergo hematopoiesis, wherein the hedgehog compound comprises a polypeptide sequence at least 80% identical to a sequence selected from SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, or a fragment thereof which binds to *patched* and induces cells to undergo hematopoiesis.